Optimal Drug and Gene Delivery in Cancer Cells by Ultrasound-Induced Cavitation

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Abstract. Background: Lack of efficient drug and gene delivery is one of the major problems of cancer chemo- and bio-therapy. This work is focused on optimization of ultrasound-induced delivery of model macromolecular anti-cancer drugs and DNA in human cancer cells. Materials and Methods: FITC dextrans simulated antisense oligonucleotides (10 kDa), antibodies (70 kDa), and genes (2000 kDa). Optimization of ultrasound frequency, intensity, duty cycle, time of irradiation, and concentration of Optison (ultrasound contrast agent) was performed. Results: Optimal parameters provided in the MCF7 cell line 73.5±3.3%, 72.7±0.9%, and 62.7±2.1% delivery of 10-kDa, 70-kDa, and 2000-kDa macromolecules, respectively, 36.7±4.9% of cell transfection, while dead cell count was only 13.5±1.6%. Statistically significant drug delivery and transfection was obtained in all tested cell lines. Conclusion: These results suggest that optimized treatment parameters provide efficient drug and gene delivery in cancer cells and could be used for further in vivo and in vitro experiments.

Lack of efficient drug and gene delivery is one of the major problems of cancer chemo- and bio-therapy (1). Different non-viral approaches have been proposed for drug and gene delivery, such as electroporation, chemical methods, liposomal delivery, gene gun mediated transfer (2-5). These techniques show a potential for drug and gene delivery, however, site-specific and efficient delivery still remains a difficult problem. Recently, novel ultrasound-mediated techniques have been proposed for drug and gene delivery (6,7). In this approach, ultrasound radiation is used to induce cavitation, which transiently increases cell membrane permeability and facilitates mechanical delivery of macromolecules and DNA in cells. In vivo application of this method is highly attractive because ultrasound radiation can be focused at almost any part of the body. Besides, it has been shown that nano- or microparticles, such as polymer particles or commercially available ultrasound contrast agents, can lower threshold of ultrasound-induced cavitation. Therefore, targeting of nano- or microparticles to specific sites and localized ultrasound-induced cavitation may lead to highly selective and efficient drug and gene delivery. This technique is non-viral, non-invasive, has excellent penetration depth, is inexpensive, and can be applied to different organs. Therefore, this technique may have an advantage over other methods for delivery of drugs and genes to specific sites including tumors.

Ultrasound-induced cavitation has been shown to deliver macromolecules and genes in cells in vitro (8-11) and in vivo (12-14) and to improve cancer chemotherapy in nude mice (15). Since ultrasound-induced drug and gene delivery has a great potential for in vivo application in cancer chemo- and bio-therapy, optimization of ultrasound-induced delivery of macromolecular drugs and DNA in cancer cells in vitro may provide a protocol which could routinely be used in anti-cancer drug design and in vivo applications. The objective of the current study was: (i) to find optimal conditions for ultrasound-induced delivery of model anticancer drugs simulating antisense oligonucleotides, antibodies, and genes, in human breast MCF7 cancer cells using an easy-to-assemble, inexpensive setup, and (ii) to test the obtained optimal parameters in six other human cancer cell lines.

Materials and Methods

Cell cultures. Seven human solid tumor cell lines: breast adenocarcinoma MCF7 (ATCC, Manassas, VA), hepatocellular carcinoma HepG2 (ATCC, Manassas, VA), cervix epithelial adenocarcinoma HeLa (ATCC, Manassas, VA), lung carcinoma A549 (ATCC, Manassas, VA), colorectal carcinoma T84 (ATCC, Manassas, VA), colon carcinoma KM20 (provided by Dr. Isaiah J. Fidler, University of Texas MD Anderson Cancer Center, Houston, TX), and...
breast adenocarcinoma SK-BR-3 (ATCC, Manassas, VA) were used in our studies. Cells were grown as monolayers at 37°C in a humidified incubator with 5% CO₂ in air. The growth medium for MCF7 cells was Minimum Essential Medium with Eagle’s salts and L-glutamine (Gibco, Invitrogen Corporation, Grand Island, NY) supplemented with 0.01 mg/ml human recombinant insulin (Gibco, Invitrogen Corporation, Grand Island, NY), 10% FBS (fetal bovine serum) from HyClone (South Logan, UT), and penicillin/streptomycin (Gibco, Invitrogen Corporation, Grand Island, NY). The growth medium for HepG2 and HeLa cells was Minimum Essential Medium with Eagle’s salts and L-glutamine supplemented with 0.1 mM non-essential amino acids, 10% FBS, and penicillin/streptomycin. A549 cells were grown in Ham’s F12K medium (Gibco, Invitrogen Corporation, Grand Island, NY) supplemented with 10% FBS and penicillin/streptomycin. The medium for SK-BR-3 cells was McCoy’s 5a with 10% FBS (fetal bovine serum) from HyClone (South Logan, UT), and penicillin/streptomycin. The growth medium for HepG2 and HeLa cells was Minimum Essential Medium with Eagle’s salts and L-glutamine (Gibco, Invitrogen Corporation, Grand Island, NY) supplemented with 1 mM sodium pyruvate (Sigma, Sigma-Aldrich, Inc., St. Louis, MO), 1X non-essential amino acids (Sigma, Sigma-Aldrich, Inc., St. Louis, MO), 1X MEM vitamins (Cellgro, Herndon, VA), 10% FBS, and penicillin/ streptomycin. The medium for SK-BR-3 cells was McCoy’s 5a with 1.5 mM L-glutamine (Gibco, Invitrogen Corporation, Grand Island, NY) supplemented with 10% FBS and penicillin/streptomycin.

Model drugs. Fluorescent FITC-dextran (Sigma, Sigma-Aldrich, Inc., St. Louis, MO) with molecular weights of 10 kDa, 70 kDa, and 2000 kDa were used as models of anti-cancer drugs simulating antisense oligonucleotides, antibodies, and genes, respectively.

Green fluorescent protein reporter plasmid (pEGFP-C1, Clontech, Palo Alto, CA) was used as a transfection marker in our experiments; pEGFP-C1 is a 4.7 kb (2,820 kDa) vector containing the Cytomegalovirus (CMV) immediate early promoter, which provides high level of gene expression in mammalian cells.

Experimental methods. MCF7 cells were subcultured 48 and 24 hours prior to irradiation. Other cell lines were subcultured 24 hours prior to irradiation. Cells were brought into suspension by washing twice with trypsin-EDTA (Gibco, Invitrogen Corporation, Grand Island, NY), allowing to stay for about 10 minutes until cells are detached, and resuspending in a fresh culture media without antibiotics to a concentration of about 10⁶ cells/ml. Then cell suspension was mixed with either FITC dextran solution (to a final concentration of 0.1% w/v) or pEGFP plasmid DNA (12.5 μg/ml) and divided in 1-ml samples. Optison (Mallinckrodt, St. Louis, MO), an ultrasound contrast agent, was added to the samples prior to irradiation to nucleate cavitation.

The cell samples were placed in 1.5-ml microcentrifuge tubes and irradiated using ultrasonic generator Sonopuls 400 (Ernst Sonopuls, Denmark). A cylindrical phantom with a diameter of 10 cm and a height of about 5 cm made from liquid plastic (item # 2116LP, M-F Manufacturing, Ft. Worth, TX) was used to minimize reflection of ultrasonic waves from the borders, provide more uniform ultrasound exposure, and simulate tumor-surrounding tissue. The microcentrifuge tubes with the samples were inserted in a hole made in the phantom close to the ultrasound transducer. Ultrasound gel was used to provide acoustic contact between the transducer and the tube as well as between the tube and the phantom. To provide more uniform ultrasound exposure, samples were rotated manually during irradiation with the frequency of about 1 Hz. The samples were exposed to different ultrasound intensities (0.5, 1, 1.5, 2, 2.5, 3 W/cm²), duty cycles (5, 10, 20%), ultrasound frequencies (1, 3 MHz), durations of irradiation (0.5, 1, 2, 5 min), and Optison concentrations (10, 50, 100, 200 ml/sample) to find optimal parameters for drug and gene delivery.

The samples with the fluorescent dextrans were kept in darkness before and after sonication to minimize FITC photobleaching. After ultrasound exposure, the samples were left at room temperature for about 30-50 minutes to allow for cell recovery. Then the samples with FITC-dextrans were washed 3 times in the growth medium without antibiotics and analyzed by flow cytometry. The samples with pEGFP DNA were transferred to 35-mm culture dishes with the growth medium without antibiotics in growing conditions, and expression of EGFP was analyzed 24 hours after the procedure.

Analysis. Intracellular loading of cells with FITC-dextrans was analyzed by confocal microscopy using Zeiss LSM510 META advanced laser scanning confocal microscope (LSCM).

To label dead cells after ultrasound exposure, we used propidium iodide (Sigma, Sigma-Aldrich, Inc., St. Louis, MO), a dye which is normally excluded from healthy cells, but penetrates into the nucleus and stains DNA of dead and dying cells. Percent of cells stained with FITC and propidium iodide was measured using a Becton-Dickinson FACScan flow cytometer (Franklin Lakes, NJ). For each sample, at least 10,000 events were recorded and analyzed by Cellquest software. Typical data obtained by flow cytometry from non-irradiated and irradiated samples are shown in Figure 1. Cellular fluorescence at the wavelengths greater than 650 nm corresponds to the propidium iodide staining, while 530-nm fluorescence corresponds to FITC labeling. Events falling in the rectangle at the right side of the plots (R1) were counted as dead cells, and the events falling in the upper left part of the plots (R2) were counted as survived FITC-labeled cells. Events, which do not fall in either of these areas, corresponded to survived non-labeled cells.

The EGFP transfection in the cells was assessed 24 hours after irradiation by FACScan flow cytometry of at least 10,000 cells for intracellular green fluorescence at the 530 nm, which indicates gene transfer and expression of EGFP in the surviving cells. EGFP expression was also conformed by fluorescence microscopy with Olympus IX70 (Melville, NY) fluorescence microscope with a standard FITC filter set.

Statistics. At least three independent samples were analyzed for each experimental condition. All data in graphs are presented as mean±SEM (standard error of the mean). Treatments were compared by one-way ANOVA. Significance was defined as p<0.05. Treated and untreated samples were compared using one-tailed Student t-test.

Results

Delivery of model macromolecular drugs in MCF7 cells. To identify optimal conditions for the delivery of drugs in cancer cells, we studied delivery of FITC-labeled dextran with molecular weights of 10 kDa, 70 kDa, and 2000 kDa mimicking antisense oligonucleotides, gene targeting vectors, and genes, respectively, in MCF7 breast cancer cells at different treatment parameters. Percent of cells loaded with
the dextran and percent of dead cells were measured over a range of different treatment parameters: total duration of irradiation, duty cycle, intensity, ultrasound frequency, and concentration of Optison (Figures 2 and 3). One of the treatment parameters was varied, while the others were fixed at 20% duty cycle, 1-min irradiation time, 3 W/cm² ultrasound intensity, and 100 µl of Optison per 1-ml sample.

At the frequency of 3 MHz, irradiation for 1 minute provided slightly better delivery than 30-second irradiation, while treatment for over 1 minute did not produce a significant change in the percentage of dead cells or cells loaded with macromolecules (Figures 2a and 3a). At the frequency of 1 MHz, optimal irradiation time was also 1 minute, since it yielded the highest percent of FITC-labeled cells (Figure 2b). Duty cycle of 20% provided the highest delivery at the frequency of 3 MHz (Figure 2c), while at the frequency of 1 MHz an optimal duty cycle was 5% (Figure 2d), probably due to higher percent of dead cells at higher values of duty cycle (Figure 2b). Ultrasound intensity of 3 W/cm² provided the highest delivery of macromolecules at both frequencies, even though the delivery did not change significantly when the intensity was over 1.5 W/cm² (Figure 2e and 2f). In the tested range, optimal Optison concentration at the frequency of 3 MHz was 200 µl, while at the frequency of 1 MHz it was 10 µl per sample (Figure 2g, 2h, 3d). Overall, ultrasound irradiation at the frequency of 3 MHz provided significantly higher delivery of model drugs and resulted in a lower percent of dead cells than irradiation at the frequency of 1 MHz (Figure 2 and 3). Therefore, the optimal delivery of macromolecular drugs in cells by ultrasound-induced cavitation in the studied range of parameters was achieved at the frequency of 3 MHz, ultrasound intensity of 3 W/cm², 20% duty cycle, irradiation time of 1 minute, and 200 µl of Optison per 1-ml sample, that provided 73.5±3.3%, 72.7±0.9%, and 62.7±2.1% of cells loaded with 10-kDa, 70-kDa, and 2000-kDa macromolecules, respectively, with 13.5±1.6% of dead cells.

To confirm intracellular presence of model drugs, MCF7 cells were studied by confocal microscopy after the treatment (Figure 4). All the three macromolecules were detected inside the cells; however, the intracellular distribution was dependent on the molecular weight of the drugs. While 10-kDa FITC-dextran was uniformly distributed inside the cells (Figure 4d), 70-kDa dextran penetrated in the nucleus, but nuclear staining was weaker than cytoplasmic (Figure 4c), and 2000-kDa dextran was visible only in the cytoplasm and not in the nuclei of treated cells (Figure 4b).

**Transfection of EGFP in MCF7 cells.** To compare delivery of model 2000-kDa macromolecular drug and actual expression of delivered DNA of similar size (2,820 kDa), transfection of MCF7 cells with EGFP by ultrasound-induced cavitation was studied at the same range of treatment parameters as the delivery of fluorescent dextrans. Figure 5 shows microscopic photograph of MCF7 cells transfected by this technique. Since the percent of dead cells measured in some of the experiments after EGFP transfection was found to be similar to that in the experiments with delivery of FITC-dextrans, it was not measured for all samples and assumed to be the same as in Figure 3. As one can see from Figures 2 and 6, the percent of transfected cells was about two times lower than the percent of cells loaded with the dextran. However, the overall trend of the dependence of EGFP transfection on treatment parameters was similar to that obtained in the experiments on...
Figure 2. Delivery of model anti-cancer drugs in MCF7 cells at different irradiation conditions. Except for the parameter which was varied in each of the experiments, irradiation conditions were 20% duty cycle, 3 W/cm² for 1 minute with 100 μl of Optison per 1-ml sample (a, c, e, g) at the frequency of 3 MHz; (b, d, f, h) at the frequency of 1 MHz. For simplicity of presentation, experiments performed at the frequency of 3 MHz are presented as open symbols, while performed at the frequency of 1 MHz are presented as solid symbols in all these Figures. One-way ANOVA indicates that dependence of percent of cells loaded with model drugs on duration of irradiation, duty cycle, ultrasound intensity and Optison concentration is statistically significant for all sizes of the drugs tested (p<0.05).
the loading with 2000-kDa FITC-dextran. In other words, optimal parameters for gene delivery in MCF7 cells were the same as for the loading of cells with macromolecules: frequency of 3 MHz, ultrasound intensity of 3 W/cm², 20% duty cycle, irradiation time of 1 minute, and 200 μl of Optison per 1-ml sample. These parameters provided 36.7±4.9% transfection of cells that survived.

Drug and gene delivery in different human cancer cells. Treatment parameters optimized in MCF7 cells were applied for delivery of model drugs and DNA in six other human cancer cell lines (Figure 7). These experiments revealed different response to ultrasound treatment, but for all tested lines, fractions of cells loaded with FITC-dextran, dead cells, and transfected cells in the treated samples were statistically significant relatively to the non-irradiated samples.

Discussion

In this study, ultrasound-induced delivery of different model drugs in human cancer cells was investigated. It was observed that macromolecules with different molecular weights penetrate similarly through plasma membrane. For example, in MCF7 cells the overall difference between the delivery rates of 10-kDa and 2000-kDa dextrans was about 20%. However, confocal microscopy revealed that intracellular distribution of the delivered macromolecules differed based on their size. Model drugs with the molecular weight of 2000 kDa did not penetrate through nuclear membrane, while 10-kDa macromolecules were uniformly distributed within the cells. Macromolecules with the molecular weight of 70-kDa can penetrate through the nuclear membrane; however, nuclear fluorescence of FITC-dextran is weaker than...
cytoplasmic, suggesting that the molecular weight of 70-kDa is close to a border size of macromolecules that can be delivered in the nucleus. These observations are consistent with results reported by Guzman et al. (9), who studied molecular uptake by DU145 prostate cancer cells exposed to 500-kHz ultrasound. Since this pattern of intracellular distribution of the molecules is consistent with limitation by size of passive diffusion through nuclear pores, these observations suggest that in our experiments macromolecules were delivered only through the plasma membrane, and then were distributed in the cell by diffusion.

Our study revealed that the various cancer cell lines have different responses to ultrasound exposure. For example, at the same treatment conditions, SK-BR-3 human breast carcinoma cells demonstrated only 3.5% of dead cells after the treatment, while HeLa human cervix epithelial adenocarcinoma cells had about 40% of dead cells. Moreover, KM20 human colorectal carcinoma cells demonstrated more than 70% loading with model macromolecular drugs, only 5% of dead cells, but relatively low percentage of cells expressing EGFP (about 10%) 24 hours after the irradiation. Most probably, these cells have an increased ability to exclude and/or degrade foreign DNA. In contrast, only about one third of T84 human colorectal carcinoma cells was loaded with FITC-dextrans, and about the same fraction of cells expressed EGFP 24 hours after the treatment, suggesting different membrane properties and low or no ability to degrade and exclude foreign DNA. Therefore, due to the variations among different cell types, ultrasound-induced drug delivery may be more or less suitable for different cell types, and further optimization of exposure conditions may improve delivery rates.

The experimental setup used in this study consists of the ultrasound exposure system and a plastic phantom. The ultrasound system Sonopuls 490 is commercially available, inexpensive, and very easy to operate. The phantom was made by heating of Plastisol. Preparation of the phantom does not require any special equipment and takes less than an hour. Once prepared, the phantom can be used for many years because it does not undergo desiccation and degradation. For that reason, the described experimental setup could be assembled in any laboratory without significant expense and in a relatively short period of time.

Our current study identified parameters of ultrasound exposure that produced highly efficient delivery of macromolecules: up to 85% of cells survived following treatment. Therefore, the drug and gene delivery protocol developed in this study could be used as a tool in anticancer drug design, to study cellular mechanisms, signaling pathways, macromolecular drug effects with a potential to mediate in vivo site-specific drug delivery or gene transfer and expression of a therapeutic gene of choice.

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Figure 6. Transfection of EGFP in MCF7 cells at different treatment conditions. Except for the parameter which was varied in each of the experiments, irradiation conditions were 20% duty cycle, 3 W/cm² for 1 minute with 100 µl of Optison per 1-ml sample. One-way ANOVA indicates that dependence of EGFP transfection on duration of irradiation, duty cycle, ultrasound intensity, and Optison concentration is statistically significant (p<0.05).

References

Figure 7. Delivery of model drugs (a), cell death (b), and transfection (c) in different human cancer cells by ultrasound with the frequency of 3 MHz, duty cycle 20%, intensity 3 W/cm² for 1 minute with 200 µl of Optison per 1-ml sample. Asterisks indicate significant differences between control and ultrasound-exposed cells (** p<0.01, *** p<0.001).


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